Mild eccentric exercise increases Hsp72 content in skeletal muscles from adult and late middle-aged rats

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Abstract The loss of muscle mass with age or sarcopenia contributes to increased morbidity and mortality. Thus, preventing muscle loss with age is important for maintaining health. Hsp72, the inducible member of the Hsp70 family, is known to provide protection to skeletal muscle and can be increased by exercise. However, ability to increase Hsp72 by exercise is intensity-dependent and appears to diminish with advanced age. Thus, other exercise modalities capable of increasing HSP content and potentially preventing the age related loss of muscle need to be explored. The purpose of this study was to determine if the stress from one bout of mild eccentric exercise was sufficient to elicit an increase in Hsp72 content in the vastus intermedius (VI) and white gastrocnemius (WG) muscles, and if the Hsp72 response differed between adult and late middle-aged rats. To do this, 30 adult (6 months) and late middle-aged (24 months) F344BN rats were randomly divided into three groups ($n=6/\text{group}$): control (C), level exercise ($16 \text{ m}\cdot\text{min}^{-1}$) and eccentric exercise ($16 \text{ m}\cdot\text{min}^{-1}$, 16 degree decline). Exercised animals were sacrificed immediately post-exercise or after 48 hours. Hematoxylin and Eosin staining was used to assess muscle damage, while Western Blotting was used to measure muscle Hsp72 content. A nested ANOVA with Tukey post hoc analysis was performed to determine significant difference ($p<0.05$) between groups. Hsp72 content was increased in the VI for both adult and late middle-aged rats 48 hours after eccentric exercise when compared to level and control groups but no differences between age groups was observed. Hsp72 was not detected in the WG

following any type of exercise. In conclusion, mild eccentric exercise can increase Hsp72 content in the rat VI muscle and this response is maintained into late middle-age.

Keywords Heat shock proteins · Aging · Skeletal muscle · Exercise · Eccentric muscle contractions · Downhill running · Muscle damage

Introduction

Cells and tissues respond to protein damaging stressors by increasing the content of highly conserved proteins known as “stress” or “heat shock proteins” (HSPs). A substantial amount of literature suggests an elevated intra-cellular HSP content confers protection to cells and tissues, including skeletal muscles (McArdle et al. 2004; Touchberry et al. 2012). While the exact mechanism(s) by which cellular protection is conferred remains unclear, it is thought that HSPs act as molecular chaperones that may minimize protein aggregation, protein denaturation, as well as reduce other “proteotoxic” related events (Noble et al. 2008). The cause of several aged-related diseases is thought to be related to protein aggregation and thus it follows that methods of elevating tissue HSP content may be beneficial in preventing these age-related diseases. In skeletal muscle, sarcopenia or the age related loss of muscle, is a major concern since muscle mass shows an inverse correlation with increased mortality and morbidity (Roubenoff 2007). NF- κ B, an important redox sensitive transcription factor, is known to play a key role in regulating skeletal muscle mass (atrophy) as well as regulating the inflammatory process (Hunter et al. 2002; Cai et al. 2004). Given that HSPs may minimize NF- κ B activation (Voegeli et al. 2008) and thereby reduce

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inflammation, the development of treatments and preventions that minimize inflammation and chronic muscle loss may be highly relevant to our aging population.

While there are many stressors known to increase HSP content, perhaps the most physiologically relevant is exercise, as it has been shown to increase the HSP content in both animal and human skeletal muscles (Thompson et al. 2001, 2003; Noble et al. 2008). In view of this, a more complete understanding of how exercise might best be used to enhance muscle HSP content and perhaps reduce inflammation and/or NF- κ B activation seems warranted. The cellular stressors associated with exercise are well documented and include increases in temperature, reactive oxygen species, an altered pH and calcium, as well as membrane and protein damage (Noble et al., 2008). Experiments using treadmill running have reported that high intensities, 24 and 27 m·min⁻¹, are necessary for HSP induction in the red vastus lateralis and white vastus lateralis muscle, respectively (Milne and Noble 2002). This suggests an intensity dependent relationship for HSP expression with exercise. While these high intensities might be suitable for young or adult animals they might not be well tolerated by older animals. Furthermore, it is known that the ability of aged cells and tissues to respond to cellular stressors appears to be reduced (Naito et al. 2001; Vasilaki et al. 2002; McArdle et al. 2004). For example, skeletal muscles from aged rats, show a reduced ability to increase Hsp72 following stimulated contractions (Broome et al. 2006). At present, it is unclear if there is a specific age at which the ability of skeletal muscle can no longer adequately increase HSP content.

Skeletal muscles are designed to shorten during contraction. However, if the resistance imposed on a muscle is greater than the force generated by the muscle, the muscle undergoes a lengthening or eccentric contraction. Examples of eccentric muscle contractions often include movements where the force of gravity must be countered such as with running downhill. Eccentric contractions are known to invoke a greater amount of muscle damage than other types of muscle contractions (Armstrong et al. 1983). Indeed, one bout of eccentric exercise has been shown to cause muscle damage as well as increase muscle HSP content (Thompson et al. 2001). In view of this, eccentric exercise may prove to be an effective method of increasing muscle HSP content without significantly increasing exercise intensity. This may be important for aged animals that are unable to tolerate the high intensities ordinarily required to increase muscle HSP content. Ultimately, these HSP elevating adaptations may be integral for minimizing sarcopenia and perhaps coping with other aged related changes (Lawler and Hindle 2011). In view of this, the purpose of the present study was to determine if there are differences in the

Hsp72 content in muscles from adult and late-middle aged rodents following a single bout of mild downhill (eccentric) running.

Methods and materials

Animals

Sixty male Fischer 344xBrown Norway rats (Harlan Laboratories Inc. Chicago, IL, USA) were used for this study. Previous research has found this breed to be effective for studying aging as these animals experience changes to skeletal muscle similar to humans (Hepple et al. 2003). Adult (A) animals (6 months, 386.0±4.5 g) and Late Middle-Age (LMA) animals (24 months, 559.3±9.1 g) were used for this study. Animals were housed at the Centre for Biological Timing and Cognition at the University of Toronto in a temperature controlled room at 21±1 °C and 67.5±3.0 % relative humidity, with reverse 12 hour light dark cycles (7:00–19:00 dark, 19:00–7:00 light) to allow the animals to be exercised during the dark cycle. Animals were housed two per cage and provided standard rat chow and tap water on an *ad libitum* basis. All experimental procedures for this study were approved by the University of Toronto's Animal Care Committee in compliance with the Canadian Council on Animal Care.

Exercise protocol

Animals were acclimatized to the treadmill with 15 minutes of walking (5–10 m/min) at 0° incline for three days. Subsequently, animals were randomly divided into the following groups: non-exercising controls (C), level exercise (L) and eccentric exercise (E). Animals in both age groups performed one hour of either level running (0° incline) or eccentric running (16° decline) at 16 m·min⁻¹. Immediately after exercise, half of the animals from each exercise and age group (L0, E0) (*n*=6) were killed and the remaining animals (*n*=6 per group) were sacrificed 48 hours post-exercise (L48, E48). Control animals were sacrificed during the same study period without being subjected to a 60-minute exercise bout. All L and E animals were exercised using a two lane rodent treadmill (Columbus Instruments, Columbus, OH, USA).

Tissue preparation

Animals were sacrificed by exsanguination from the apex of the heart under anesthesia (2.5 % isoflurane inhalation with oxygen). The white gastrocnemius (WG) and vastus intermedius (VI) were rapidly dissected and the mid-belly of each muscle was cut transversely to the long axis of the

muscle, mounted in OCT medium on cork and frozen in isopentane cooled by liquid nitrogen. The remaining portions of each muscle were frozen in liquid nitrogen and stored at -80°C for subsequent biochemical analyses.

Histochemistry

Transverse muscle samples were cyrosectioned ($10\text{ }\mu\text{m}$) (Leica 3500S, Germany) at -20° and subsequently stained with hematoxylin and eosin (H&E) to assess muscle damage. Stained sections were visualized at 20x magnification using a Zeiss Axio-Imager microscope (Zeiss, Oberkochen, Germany). To provide an estimate of muscle damage based on immune cell infiltration, stained nuclei from 20 adjacent muscle fibres per cross section were counted manually using ImageJ Analysis Software (National Institute of Health, USA). This number of muscle fibres was selected for counting to provide a sample area large enough to reduce variability between samples, yet small enough to be a subsection of the muscle cross section. Muscle sections from controls were also assessed to determine resting cell and muscle nuclei content, thereby illustrating exercise induced changes in immune cell presence as an indication of muscle damage.

SDS-PAGE and Western blotting

Muscle portions ($\sim 50\text{ mg}$) were homogenized in 15x volume of 600 mM NaCl and 15 mM Tris (pH 7.5) and protein concentration determined using the method described by Lowry et al. (1951). One-dimensional sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (1970) using a 10 % separating gel. Samples were subsequently electrophoresed using a Bio-Rad Power Pac 1000 (Bio-Rad, Mississauga, Ontario) at 60 volts until the dye front had moved into the separating gel (~ 45 minutes) followed by 100 volts until the dye reached the bottom of the separating gel.

Proteins were transferred to nitrocellulose membrane paper ($0.22\text{ }\mu\text{m}$ pore size, Bio-Rad, Mississauga, Ontario) using the Bio-Rad miniprotein II gel transfer system at 40 volts for 3 hours. Once the protein transfer was complete, nitrocellulose paper (blot) was blocked in Blotto (5 % non-fat skim milk powder in Tris Buffer Saline (TBS, 500 mM NaCl and 20 mM tris [pH 7.5]) for one hour to overnight. Nitrocellulose membrane papers were rinsed twice in TTBS (TBS with 0.05 % Tween-20) and incubated for 3 hours at room temperature with a polyclonal antibody specific for Hsp72 (SPA-812-F, Stressgen, Ann Arbor, MI, USA), diluted 1:1000 in TTBS with 2 % non-fat skim milk powder. After 2x5 minutes washes in TTBS, blots were placed in a 2 % non-fat skim milk powder solution with secondary

antibody (#7054 goat anti-rabbit immunoglobulin-G conjugated to alkaline phosphatase) (Cell Signalling, Pickering, Ontario, Canada) at a 1:3000 dilution in TTBS supplemented with 2 % NFDM powder for 1 h. Hsp72 bands were then visualized on blots washed 2x5 minutes in TTBS, 1x5 minutes in TBS, and immersed in carbonate buffer (100 mM Na_2CO_3 , 1 mM MgCl_2 , pH 9.8) containing one milliliter of 3 % (w/v) p-nitro-blue-tetrazolium chloride p-toluidine salt in 70 % N,N-dimethyl-formamide (DMF) and one milliliter of 15 % (w/v) 5-bromo-4-chloro-3-indolyl phosphate in 100 % DMF. After development, the blots were washed in distilled H_2O , dried and scanned.

Quantification of bands from immunoblots was performed using ImageJ Analysis Software (National Institute of Health, USA). To compare the content of protein in each lane, band intensity was measured and normalized to a percentage of the Adult Control band on each blot to allow for comparison between groups. Normalized values from each blot were averaged to determine overall differences between groups for Hsp72.

Statistical analyses

A nested ANOVA design was used for this study, where exercise condition was nested within the factor age. A Tukey's honestly significant difference post-hoc analysis was used when appropriate. Statistical analysis was performed using R version 2.14. Data are displayed as mean $\pm$ standard error of the mean (SEM). Differences between groups were considered significant at $p < 0.05$. Descriptive statistics for animal weight and immune cell infiltration counts are shown as mean $\pm$ standard deviation (SD).

Results

Physical characteristics

The body mass of adult and late middle aged animals was $386.0 \pm 4.5\text{ g}$ and $559.3 \pm 9.1\text{ g}$, respectively (Table 1). Not surprisingly, the late middle-aged animals were significantly heavier than adult animals ($p < 0.05$). However, there were no mass differences between exercise groups of the same age group.

Muscle damage

Following eccentric exercise, analyses of hemotoxylin and eosin stained muscle cross sections showed immune cell infiltration in the VI and to a muscle lesser extent in the WG muscles (Fig. 1). A group of 20 adjacent representative muscle fibres was examined and the immune cells surrounding these fibres were counted for both age groups and

Table 1 – Animal weights displayed by age group and exercise condition

Animal Weight (g)					
	C	L0	L48	E0	E48
A	380.8±20.1	374.0±31.6	386.0±16.8	387.6±16.8	401.5±24.9
LMA	526.7±58.5	545.6±46.9	572.1±34.0	583.9±55.8	568.4±45.7

Animal weights in grams for Adult (A) and Late middle-aged (LMA). Control (C), Level (L), Eccentric (E) at time 0 and 48 hours post exercise. Mass is provided as mean ± SD.

exercise conditions (Table 2). The VI muscle had the highest number of immune cells for all samples analyzed when compared to the WG. The muscles removed directly following eccentric exercise had the highest infiltration of immune cells for both VI and WG muscles, while the VI muscles taken directly following eccentric exercise groups having the highest cell count for each age group. These results suggest eccentric exercise was more damaging than level exercise.

Muscle Hsp72 content

To determine Hsp72 content, muscle proteins from the VI and WG were subjected to SDS-PAGE and Western blot analyses. In the VI muscle, exercise did not elevate Hsp72 content compared to adult controls. The Hsp72 content measured in the late middle aged animals subjected to downhill running and allowed to recover for 48 hours was significantly higher than late middle aged animals subjected to downhill running and allowed to recover for 0 hours and

late middle aged animals subjected to level running and allowed to recover for 48 hours ($p<0.05$). In adult animals subject to downhill running and allowed to recover for 48 hours, Hsp72 was significantly higher than late middle-aged animals that had no recovery ($p<0.01$). At 48 hours post exercise, eccentric exercise increased muscle Hsp72 content in both adult and late middle-aged groups over level exercise (Fig. 2b). Since the effect of exercise and age compared to controls was unclear, these data were pooled to create a larger sample size. Subsequent analyses determined at 48 hours post-eccentric exercise, muscle Hsp72 content was significantly higher compared to all other age groups ($p<0.02$) (Fig. 2c). Hsp72 was undetectable in the WG muscles in all groups examined.

Discussion

An increased Hsp72 content has been shown to provide protection to skeletal muscles allowing them to adequately

Fig. 1 - Eccentric exercise causes damage to muscle fibres in the VI and WG muscles. Dark stained features are nuclei of muscle fibres or immune cells. Muscle damage from exercise is evident by immune cell infiltration. Panels **a** and **b** show the VI muscles of adult control and adult 48 hours after eccentric exercise, respectively. Panels **c** and **d** show the WG muscles of adult control and adult 48 hours after eccentric exercise, respectively

Table 2 – Increased immune cell infiltration following eccentric exercise

Muscle		Immune Cell Infiltration				
		C	L0	L48	E0	E48
VI	A	51±10	128±9	83±14	195±30	158±30
	LMA	69±6	128±13	–	165±21	–
WG	A	55±8	61±3	84±7	83±22	62±14
	LMA	53±4	73±7	–	145±4	–

Total immune cell infiltration from the area surrounding 20 adjacent representative muscle fibres from Adult (A) and Late middle-aged (LMA) animals ($n=2-3$). Control (C), Level (L), Eccentric (E) at time 0 and 48 hours post-exercise. – Indicates samples were lost through a freezer malfunction. Values are mean $\pm$ SD.

cope and adapt to increased physiological stress and increased temperature (Tupling et al. 2008). Although a number of HSPs are thought to play roles in cellular protection, Hsp72, the inducible member of the Hsp70 family, appears to be the most relevant and the most extensively studied. In the present study, Hsp72 content was examined in two muscles from adult and late middle-aged rats following downhill and level running. The main findings from this study are: i) mild intensity eccentric running can induce an increase in Hsp72, ii) Hsp72 accumulation is greater following downhill, eccentrically biased exercise than following level exercise and iii) both adult and late middle-aged rats increased muscle Hsp72 content in response to downhill running (eccentric exercise).

The present study indicates different amounts of damage and Hsp72 content between the VI and WG muscles in response to acute mild eccentric exercise. Muscle cross-sections (Fig. 1) and immune cell counts (Table 2), show the VI muscle from adult animals sustained greater damage when compared to the WG muscle. These findings agree with others (Armstrong et al. 1983) where muscle damage following either level or eccentric running was found to be significantly elevated at 48 hours post exercise in the VI, but not in the WG muscle.

In the present study, hemotoxylin and eosin staining of muscle cross-sections of the VI, suggests eccentric exercise in the form of downhill running provided a more intense and damaging stimulus than level running. This muscle damage appeared to increase Hsp72 content in the VI muscle, since the level running groups did not show an elevation in Hsp72 content. As mentioned, the major difference between level and downhill running used in the present study is the type of muscle contraction. When compared to running on a level plane, running downhill requires greater eccentric contractions to counteract the increased gravitational force. As a result, downhill running elicits a greater amount of muscle damage (Armstrong et al. 1983; Touchberry et al. 2012). Given that the mass of the late to middle aged animals was

significantly greater than the adult animals they might be expected to have to cope with greater gravitational forces and thus experience a greater level of muscle damage as well as a greater Hsp72 accumulation. However, there were no differences in VI muscle Hsp72 content between adult and late middle-aged animals at 48 hr after eccentric exercise suggesting the level of stress imposed by running was not greater.

Hsp72 was not detected in the WG muscles from adult or late-middle aged animals. While the exact reason for the lack of detecting Hsp72 cannot be determined from the present study, it may be that the WG muscle was not sufficiently recruited at the selected mild pace (16 m/min) and thus was not metabolically or physically stressed. In support of this, the response during level treadmill running has showed the white portion of the vastus lateralis only increased muscle Hsp72 content following one hour of level running when intensity was at 27 m·min⁻¹ (Milne and Noble 2002). Motor unit recruitment of fast fibres from rat gastrocnemius muscle follows the standard motor unit size recruitment principles and thus animals have a low level of fast fibre activation when running at 12 m·min⁻¹ and the highest when running at 30 m·min⁻¹. Given that this exercise protocol involved a running speed of 16 m·min⁻¹ it remains probable that animals were running at a speed that did not recruit fast fibres. The lack of fast fibre recruitment at this exercise intensity, in the predominantly fast fibre comprised WG, would preclude any damage and hence need for an elevated Hsp72 content.

Skeletal muscle Hsp72 content following exercise was not influenced by age, which suggests the ability to up regulate Hsp72 is maintained into late middle-age. Given that the late middle-aged animals were 24 months old and showed an elevated Hsp72 content following downhill running, it shows that these animals are capable of increasing the purportedly protective Hsp72 protein. It should be noted that the elevated Hsp72 content detected in these muscles may represent a response to muscle damage and thus not be available for muscle protection. Whether muscles in this condition are more or less susceptible to subsequent stressors remains to be determined. Regardless, adult and late middle-aged animals responded similarly to eccentric exercise with an increased VI muscle Hsp72 content at 48 hours post-exercise (Fig. 2). This suggests the ability to regulate the synthesis of muscle Hsp72 is maintained through late middle-age in rat VI muscle.

In conclusion, the present study provides evidence that a single bout of mild eccentric exercise can increase muscle Hsp72 content in the VI muscles of both adult and late-middle aged rats. This compensatory increase appears to be related to the muscle damage

Fig. 2 – HSP72 content in the VI from LMA animals is significantly increased 48 hours following eccentric exercise. Panel **a** shows a representative Western blot for HSP 72 from all groups. Control (C), Level (L), Eccentric (E) at time 0 and 48 hours post exercise for both adult and late-middle aged rats. Panel **b** is a graphical representation of

scanned band intensity normalized to Adult Control. + Significantly different from LMAE-0 and LMAL-48 ($p < 0.05$). * Significantly different from LMAE-0 ($p < 0.05$). Panel **c** is the pooled results of A and LMA groups normalized to Control. * Significantly different from all other groups ($p < 0.02$). Values are presented as means $\pm$ SEM

following eccentric or lengthening contractions. Given that eccentric contractions can be attained without the complications of high intensity exercise, it suggests that

this type of exercise might be a practical intervention to provide skeletal muscle protection for late middle-aged humans.

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